system at the stage of cytochrome *c* reductase; the diaphorase activity is not affected.

7. Thiol compounds react chemically with fluoropyruvate, thereby preventing its inhibitory activity. The reaction products show characteristic spectra in the ultraviolet region.

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The Metabolism of Short-chain Fatty Acids in the Sheep

4. THE PATHWAY OF PROPIONATE METABOLISM IN RUMEN EPITHELIAL TISSUE*

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Propionic acid is metabolized by the epithelial tissue of the rumen, and it is likely that a substantial part of the propionic acid produced by microbial activity in the rumen is metabolized by this tissue. In a previous paper in this series (Pennington, 1954) it was shown that the rate of metabolism of propionic acid by sections of sheep-rumen epithelium increased with the concentration of carbon dioxide/bicarbonate in the system. It was accordingly suggested that propionic acid (or propionyl coenzyme A) may be carboxylated by addition of carbon dioxide. The possibility that the observed effect was due to a recognized fixation reaction, namely addition of carbon dioxide to pyruvate formed from propionate, appeared less likely from certain other evidence which was presented. Conversion of propionate into succinate in liver and kidney would provide an explanation for the results of the Cleveland investigators (Lorber, Lifson, Sakami & Wood, 1950; Shreeve, 1952), who found that the α- and β-carbon atoms of propionate were completely randomized during the formation of glycogen or acetyl groups. On the other hand, Wisconsin workers (Huennekens, Mahler & Nordmann, 1951; Mahler & Huennekens, 1953) postulate a pathway of α-oxidation via lactate in liver.

The present paper is concerned with further investigations with carbon-14, into the pathway of metabolism of propionate in sheep-rumen epithelial tissue. The results obtained support the theory that succinate is formed from propionate and carbon dioxide. In addition, some studies were made on the 'antiketogenic' action of propionate with this tissue and a mechanism is suggested to account for the suppression of ketone-body formation by propionate.

* Part 3: Annison & Pennington (1954).

Preliminary communications of this work were submitted to the Biochemical Society (Pennington & Sutherland, 1954, 1955).

EXPERIMENTAL

Sections of rumen epithelial tissue were prepared and incubated in Ca^{2+}-free Ringer solution buffered with phosphate or bicarbonate or both and containing appropriate substrates (total vol., 10 ml.), as previously described (Pennington, 1954). The NaCl concentration of the medium was increased to make it isotonic with sheep blood (Aldred, 1940). The analyses performed on the media were corrected for retention of metabolites by the tissue (Pennington & Sutherland, 1956). For some of the experiments tissue was removed immediately after killing, from sheep slaughtered at the Institute, and was kept before use in ice-cold Ringer–phosphate solution continuously aerated with oxygen. In other experiments slaughterhouse tissue was used, involving slight delays in removal from the animal after slaughter.

Analytical methods

Propionic acid was determined by steam distillation in a Markham still and titration (Pennington, 1952). No other volatile fatty acids are produced when propionate is metabolized by this tissue (Pennington & Pfander, unpublished results). Total ketone bodies, pyruvic and lactic acids were determined as described elsewhere (Pennington & Sutherland, 1956).

Extraction of organic acids

Organic acids were extracted with ether from the incubation media by efficient Kutscher–Steudel liquid–liquid extractors. For a general description of this apparatus see Cohen (1945). The bottom of the funnel was turned up and fused on to a sintered-glass disk to break up the ether bubbles, and the funnel was lengthened to 40 cm. so that the head would be sufficient to force the ether through the sintered glass. The lower part of the tube, containing the aqueous phase, was kept cool in a bath of running water to increase the partition coefficient of the acids. With these units succinic acid was completely extracted within 30 min. The minimum time required for the extraction of citric acid was not determined, but extraction was complete after 24 hr. Normally the sample, in a volume of 20 ml., was acidified and deproteinized with 4 ml. of 15% HPO_3. Before extraction, 20 g. of $MgSO_4,7H_2O$ was added. Any variations from this procedure are described in the text. After extraction, the bulk of the ether was distilled off, the last traces were removed by a current of dry air, and the mixture of acids was taken up in $CHCl_3$–*tert*.-pentanol (90:10, v/v) for chromatography.

Chromatography of organic acids

Carboxylic acids were separated by chromatography on acid silica gel columns, a method introduced by Isherwood (1946) and used with modifications in procedure by other workers (Frohman, Orten & Smith, 1951; Marshall, Orten & Smith, 1949). Much preliminary work was required to find suitable conditions for the satisfactory separation of all the acids likely to interest us, particularly lactic and succinic acids. Finally, the procedure described below was adopted.

Preparation of gel. The best separation of carboxylic acids was obtained when the gel was prepared by a procedure based closely on that of Gordon, Martin & Synge (1943) and Tristram (1946). Sodium silicate (200 ml.) was dissolved in 200 ml. of distilled water, the solution cooled, and crushed ice, made from 200 ml. of distilled water, added together with methyl orange (2 ml. of a 0·1% solution). Cooled 10 N-HCl was added in a fine stream with vigorous stirring. When the indicator remained pink for 3 min. a further 40 ml. of 10 N-HCl was added and the mixture stood for 3 hr. with occasional stirring. It was then filtered by suction and the precipitate washed on the funnel with 2 l. of 5 N-HCl; the suction was adjusted so that this took at least 1 hr. to run through. Finally the gel was washed with distilled water until the washings were Cl^- free. It was then mixed with 1500 ml. of 0·2 N-HCl, stood 3–4 days with occasional stirring, filtered, and washed until Cl^- free. After a short period of drying at 105° all lumps were ground out and the powder was dried for 24 hr. at 105° and stored in airtight containers.

Chromatographic procedure. A sample (1·5 g.) of the silica was ground rapidly in a mortar with dil. H_2SO_4 (see below), the mixture transferred to a stoppered tube and vigorously shaken for 5 min. in a mechanical shaker with 20 ml. of dry $CHCl_3$; the $CHCl_3$ used in all these experiments contained 1% of ethanol added as a stabilizer. The slurry was poured into a tube 50 cm. × 0·7 cm. internal diameter, air bubbles were removed by rapid stirring with a thin glass rod, and 50 ml. of $CHCl_3$ was run through to pack the column. The sample, dissolved in 1 ml. of $CHCl_3$–*tert*.-pentanol (90:10, v/v), was added to the top of the column, followed by 1 ml. of $CHCl_3$ when the sample had run in. The top of the tube was attached to a mixing reservoir of the type described by Donaldson, Tulane & Marshall (1952), which delivers a liquid of progressively changing composition on to the column. The lower chamber of the reservoir, the volume of which was 165 ml. up to the side arm, contained $CHCl_3$ equilibrated with 0·25 N-H_2SO_4. In the upper vessel was placed a mixture of 4 vol. of equilibrated $CHCl_3$ and 1 vol. of *tert*.-pentanol. Thus the solvent flowing through the column contained a gradually increasing proportion of pentanol. Fractions (2 ml.) were collected and titrated with 0·004 N-KOH in methanol. Dry, CO_2-free air was bubbled through the liquid from 1 min. before commencing the titration. Cresol red was used as indicator.

Fig. 1 shows a typical chromatogram of acids which were separable. The formic acid peak overlapped those of pyruvic and fumaric acids; β-hydroxybutyric acid followed on and slightly overlapped fumaric acid, whilst malonic acid followed and markedly overlapped α-oxoglutaric acid. The initial rate of flow was normally about 50 ml./hr.; if it exceeded about 60 ml./hr. the peaks were noticeably less sharp. The rate of flow was governed critically by the moisture content of the gel, increasing with increase in the latter. This varied slightly with different batches and it was necessary to ascertain with each batch the volume of dil. H_2SO_4 to be mixed with 1·5 g. of gel to produce a satisfactory flow rate. This was usually between 1·2 and 1·4 ml., and the concentration was adjusted so that it contained in all cases 0·375 m-equiv. of acid.

In some cases it was desired to determine lactic and malic acids on the fractions of the corresponding peaks from the column, following titration. The fractions were extracted with an equal volume of 0·02 N-NaOH. In the case of lactic acid the alkaline solution was then extracted twice with

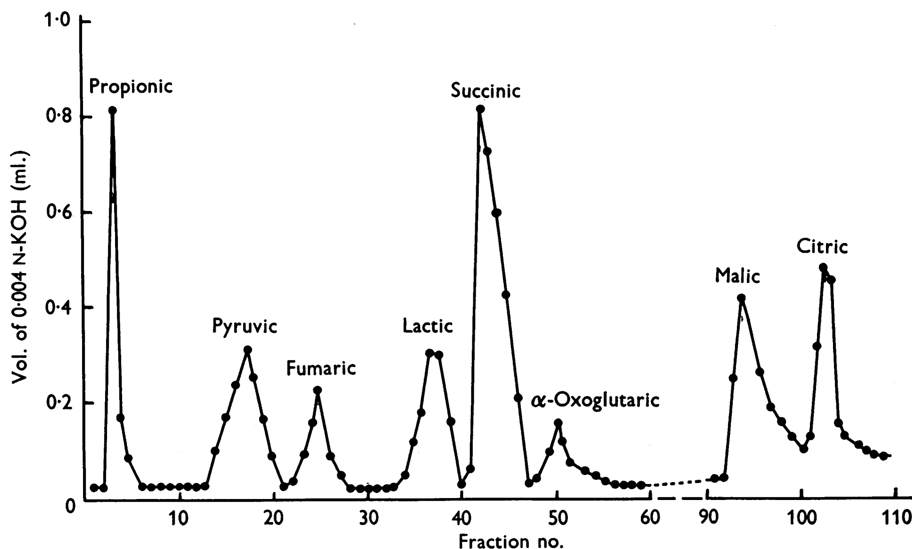

Fig. 1. Chromatogram of acid mixture on acid silica-gel column. Gradient elution with chloroform-*tert.*-pentanol was used, as described in the text.

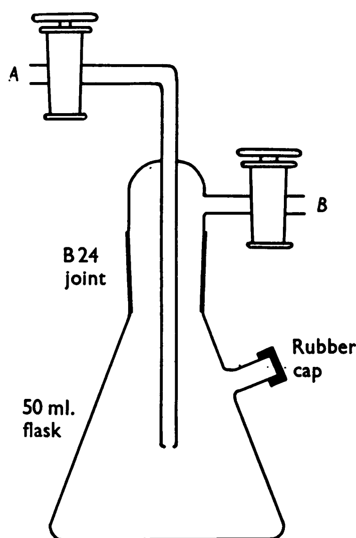

Fig. 2. Unit for isotope studies with tissue sections.

equal volumes of ether in order to remove pentanol, which was found to inhibit the development of the colour in the determination of lactic acid with *p*-hydroxydiphenyl.

The recovery of lactic acid after chromatography was always low (about 80%). The values given in the tables for total radioactivity in the lactate have been corrected accordingly.

For an unexplained reason fluorimetric determination of malic acid (Hummel, 1949) on fractions from the malate peak gave values only about one-quarter of those expected from the titration figures. This was so, both with malate produced by the tissue and when pure solutions of malate were passed through the silica-gel column.

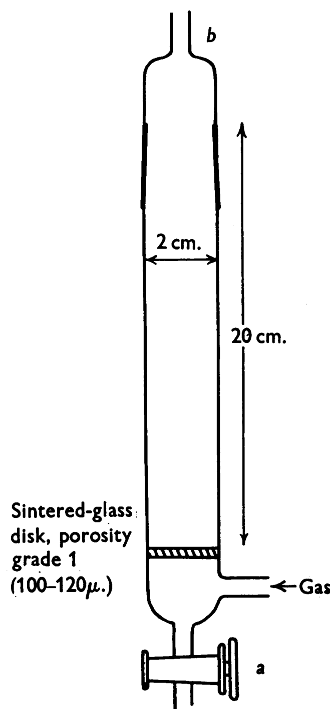

Fig. 3. Gas-absorption vessel.

Radioactivity procedures

For experiments involving measurement of the production or fixation of $^{14}CO_2$ by the tissue a special unit consisting of a flask and wash-bottle head fitted with taps was used (Fig. 2). After addition of the tissue and medium the flask

was gassed for 10 min. through A with the appropriate gas mixture. After closing the taps a small amount (1–5 μmoles) of labelled substrate or $NaH^{14}CO_3$ of high specific activity (about 1 μc/μmole) was injected through the rubber cap. To assay $^{14}CO_2$ after incubation of the flask, acid was injected through the cap, CO_2-free air passed in at A and the gas from B passed through 50 ml. of 0·1 N-NaOH (CO_2-free) in an absorption vessel of the design shown (Fig. 3). The maximum allowable gassing rate was not ascertained, but complete absorption of CO_2 was obtained with a rate of 10 ml./min. Following absorption the alkali was readily forced out through tap a by applying a slight pressure at b and the tube washed out with a little water. To the alkali solution was added 6 ml. of N-NH_4Cl and after heating nearly to boiling at least twice the expected amount of 0·2 M-$BaCl_2$ required for precipitation of the carbonate, and not less than 2 ml., was added. After cooling, the precipitate was filtered off on a sintered-glass filter, washed successively with cold water (about 10 ml.), acetone and ether, and dried at 100° for 1 hr. and weighed.

When propionic acid was present in the flask there was a slight carry-over and absorption of propionate on the $BaCO_3$ precipitate. The addition of 100 μmoles of unlabelled propionate before precipitating the $BaCO_3$ prevented appreciable contamination of the $BaCO_3$ with radioactive propionate.

A portion of the dried $BaCO_3$ precipitate was ground with ethanol and the slurry transferred to a weighed nickel planchet (1·5 cm. in diameter) and spread uniformly with a thin glass rod. After drying and weighing, the radioactivity was measured with an end-window Geiger counter. The activity was corrected to zero thickness in the usual manner with a self-absorption curve constructed from samples of a standard $Ba^{14}CO_3$ of varying weight. In some experiments a portion of the Na_2CO_3–NaOH solution was precipitated with $BaCl_2$ in a centrifuge tube. The precipitate was washed by centrifuging and transferred completely to the planchet.

The radioactivity in the fractions from the silica-gel columns was measured after adding a slight excess of KOH and evaporating the solution in a current of air while warming the tube. When the volume was sufficiently small the liquid was transferred to a planchet and dried on a hot plate. The weight of material present was normally too small to necessitate a self-absorption correction.

The labelled propionate and fumarate used as substrates in the experiments were all obtained from the Radiochemical Centre, Amersham, and their counting activity under our conditions was measured after evaporation of small quantities on planchets.

RESULTS

Chemical analysis showed that lactate was a major product of the metabolism of propionate by rumen epithelial sections. This was confirmed by silica-gel chromatography, which also failed to reveal the

Table 1. *Metabolism of* [carboxy-^{14}C]*propionic acid by sheep-rumen epithelium*

Tissue (2 g., from slaughterhouse sheep) was incubated for 3 hr. at 39·5° in Ringer–phosphate–bicarbonate containing the additions indicated (as sodium salts). Gas phase: $O_2 + CO_2$ (95:5, v/v).

Expt. no.	Substrate	Substrate utilized* (μmoles)	Lactate formed* (μmoles)	Substrate carboxyl-C in lactate† (μatoms)	Substrate carboxyl-C in CO_2† (μatoms)
1	[*carboxy*-^{14}C]Propionate (100 μmoles)	70·8	29·8	6·10	58·2
	—	61·0	29·3	4·16	57·2
	—	—	3·2	—	—
2	[*carboxy*-^{14}C]Propionate (100 μmoles)	—	11·9	2·02	31·0
	—	—	2·5	—	—
3	[*carboxy*-^{14}C]Fumarate (500 μmoles)	—	13·2	3·56	10·2
	—	—	4·4	—	—

* By chemical analysis. † Total counts/min. in lactate or $BaCO_3$ divided by counts/min./μmole of substrate.

Table 2. *Fixation of carbon dioxide during metabolism of propionate, pyruvate and fumarate by epithelium*

Tissue (from slaughterhouse sheep) was incubated for 3 hr. at 39·5° in Ringer–phosphate–bicarbonate containing the substrates shown. Gas phase: $O_2 + CO_2$ (95:5, v/v).

Expt. no.	Wt. of tissue (g.)	Substrate: Nature	Substrate: Amount (μmoles)	Substrate utilized (μmoles)	Lactate formed (μmoles)	CO_2 fixed (μmoles)
1	3	Propionate	100	60·1	—	25
	—	—	—	—	—	0·2
2	3	Propionate	100	53·4	33·2	30
	1·5	Pyruvate	100	37·5	20·4	1·7
	1·5	—	—	—	—	0·5
3*	2	Propionate	200	—	32·4	19·4
	2	Fumarate	500	—	11·2	1·23
	2	—	—	—	4·4	0·53

* Tissue from the same animal as used for Expt. 3, Table 1.

presence of any other ether-soluble organic acid after incubation of the tissue with propionate. Table 1 shows the results of experiments in which [*carboxy*-^{14}C]propionic acid was metabolized and the amount of isotope appearing in the carbon dioxide and lactic acid was measured. It may be seen that the greater part of the isotope in the propionate utilized can be accounted for as carbon dioxide. In line with this, the specific activity of the lactate formed was only a fraction of that of the propionate. The difference would be even greater if the radioactivity of the lactate were corrected for the incorporation of $^{14}CO_2$ formed from the propionate (see Table 2). It is improbable that this was due to dilution by unlabelled lactate from other sources, since the endogenous-lactate production was low. In a similar experiment with carboxyl-labelled fumarate (Expt. 3) the specific activity of the lactate formed was low also, but relatively higher than that of the lactate formed from propionate. In this experiment unlabelled lactate equivalent to ten times the amount of lactate in the medium was added before ether extraction, and the observed specific activity corrected accordingly. Otherwise the lactate peak on the chromatogram was swamped by the large quantity of fumarate present.

In order to determine whether the metabolism of propionate is accompanied by fixation of carbon dioxide, as seemed likely from the effect of carbon dioxide upon the rate of metabolism of propionate, propionate was metabolized in the presence of $^{14}CO_2$ (Table 2). For comparison, similar experiments were carried out with pyruvate and fumarate.

The quantity of $^{14}CO_2$ fixed was computed from the total radioactivity in a 24 hr. ether extract of the acidified medium and tissue washings. There was negligible activity remaining in the medium after ether extraction, and the tissue itself had no measurable activity after drying. Within the limits of error all of the activity in the ether extract could be accounted for by the activity of the lactic acid. Degradation of the lactic acid produced from propionate in Expt. 2 with chromic acid (Aronoff, Barker & Calvin, 1947) showed that essentially all of the radioactivity was in the carboxyl group.

The fact that a considerable amount of carbon dioxide is 'fixed' during the metabolism of propionate, whilst there is relatively a very small fixation when pyruvate or fumarate are metabolized, could reasonably be explained by addition of carbon dioxide to propionate to form succinate and ultimate conversion of the succinate into lactate. To test this hypothesis, malonate was used to block the further metabolism of succinate formed from labelled propionate and from propionate in the presence of labelled carbon dioxide. It was previously shown (Pennington, 1954) that succinate is

Table 3. *Formation of labelled succinate in presence of malonate from* [carboxy-^{14}C]*propionate and from propionate and* $^{14}CO_2$

Tissue (2 g. in Expts. 1 and 2; 3 g. in Expts. 3 and 4) was incubated for 3 hr. at 39·5° in Ringer–phosphate–bicarbonate. Gas phase: $O_2 + CO_2$ (80:20, v/v) in Expts. 1–3; $O_2 + CO_2$ (95:5, v/v) in Expt. 4. The CO_2 was labelled in (*a*) and (*b*) in each experiment. All flasks contained sodium malonate (0·01 M in Expt. 3; 0·02 M in Expts. 1, 2 and 4). The tissue in Expts. 1 and 2 was obtained from sheep killed at the slaughterhouse; that obtained in Expts. 3 and 4 was from sheep slaughtered at the Institute and was removed rather more quickly after killing.

Expt. no.	Addition	Propionate carboxyl-C in CO_2 (µatoms)	Succinate produced (µmoles)	Incorporation of labelled carbon from propionate or CO_2 into succinate (µatoms)	(% in additional succinate*)	Calc. value for % incorporation†	Propionate uptake (µmoles) (calc.)	Lactate produced (µmoles)
1 *a*	—	—	2·72	0·06	—	—	—	—
b	400 µmoles of Na propionate	—	6·66	1·91	47	—	—	—
c	400 µmoles of Na [*carboxy*-^{14}C]propionate	16·7	6·40	2·34	64	56	19·0	—
2 *a*	—	—	2·35	0·02	—	—	—	—
b	400 µmoles of Na propionate	—	4·43	1·41	67	—	—	—
c	400 µmoles of Na [*carboxy*-^{14}C]propionate	13·2	4·53	1·05	48	53	14·3	—
3 *a*	—	—	2·84	0·18	—	—	—	6·8
b	400 µmoles of Na propionate	—	10·95	5·65	67	—	—	30·3
c	400 µmoles of Na [*carboxy*-^{14}C]propionate	44·3	11·29	5·94	70	66	54·3	27·2
4 *a*	—	—	3·08	0·19	—	—	—	2·9
b	400 µmoles of Na propionate	—	19·14	9·15	56	—	—	5·4
c	400 µmoles of Na [*carboxy*-^{14}C]propionate	54·2	19·82	9·30	56	59	64·1	6·1

* Specific activity (counts/min./µmole) of additional succinate formed from propionate/specific activity of propionate or CO_2. The assumption is made that endogenous succinate production is unaltered when propionate is metabolized.

† See Discussion.

Table 4. *Degradation of succinate produced by metabolism of propionate*

The fractions constituting the succinate peak from each chromatogram in Expt. 4, Table 3, were combined, after radioactivity measurements, and the succinate content was checked manometrically by succinic oxidase. The resulting fumarate–malate mixture was degraded with acid permanganate. Acetaldehyde from the degradation contained no detectable radioactivity.

Substrate	Succinate in chromatogram peak* By titration (μmoles)	By succinic oxidase (μmoles)	Total activity in chromatogram peak (counts/min.)	Activity of CO_2 from degradation (counts/min.)
Propionate, $^{14}CO_2$	11·8	12·4	5581	6011
[*carboxy*-^{14}C]Propionate, CO_2	10·6	7·8	5594	6348

* A portion of the medium was extracted and chromatographed; hence these figures do not correspond with those in Table 3, which represent total succinate production.

formed by this tissue when propionate is metabolized in the presence of malonate. The results of several experiments are given in Table 3. Since it would probably be difficult to separate any succinate formed from the large quantity of malonate present, owing to the closeness of their R_F values the malonate was destroyed by acid permanganate before extraction of the medium with ether. The formic acid thus formed was removed when the ether was evaporated off. Any α-oxoglutarate present would be oxidized to succinic acid by this treatment; this acid is not likely to be present in appreciable amounts. The succinate was isolated by silica-gel chromatography and its radioactivity determined. It may be seen that poorer yields of succinate were obtained with tissue from slaughter-house sheep, presumably because of the inevitable delay in removal of the rumen after killing, or as a result of the starvation of the animals before slaughter.

The succinate isolated in all cases accounted for only a fraction of the propionate metabolized, owing presumably to incomplete blocking of succinate oxidation at the malonate concentrations used. (Concentrations of malonate greater than 0·02 M were not used since the amount of propionate disappearing was sharply decreased, being negligible with 0·08 M malonate.) The succinate formed, however, was heavily labelled; the specific activity, after correcting for the endogenous succinate, averaged over one-half that of the propionate or carbon dioxide. Of particular interest is the fact that the specific activity of the succinate relative to that of the starting material was of the same order, and in two of the experiments closely similar, whether the isotope came from the propionate or the carbon dioxide.

Lactate formation was markedly decreased by 0·2 M malonate (Expt. 4).

The identity of the succinate in these experiments was deduced initially from the position of the peak on the chromatogram together with its stability to acid permanganate. The ratio of radioactivity to titration figure of all the fractions constituting the peak was approximately constant. In Expt. 1 succinic acid was added to a portion of the ether extract before chromatographing; the specific activity of the fractions in the peak was again constant and diluted by the expected amount. A further check was provided by two other experiments in which the fractions from the peak, after drying and counting, were redissolved, pooled and ether-extracted, and the succinic acid was determined manometrically with a sheep-heart succinic oxidase preparation (Cohen, 1945). The resulting mixture of fumarate and malate, after removal of protein with metaphosphoric acid, was oxidized with acid permanganate (Wood, Werkman, Hemingway & Nier, 1941). The carbon dioxide produced was counted as barium carbonate and the acetaldehyde as the 2:4-dinitrophenylhydrazone. Table 4 indicates that all of the activity of the succinate was located in the carboxyl groups.

Table 5. *Specific activity of succinate and lactate formed during the metabolism of propionate in the presence of malonate*

Tissue (3 g.; from Institute sheep) was incubated for 3 hr. at 39·5° in Ringer–phosphate–bicarbonate containing sodium propionate (400 μmoles) and sodium malonate (0·01 M). Gas phase: $O_2 + CO_2$ (95:5, v/v). [*carboxy*-^{14}C]-Propionate was used in the first run and $^{14}CO_2$ in the second.

	Specific activities (counts/min./μmole) Propionate	CO_2	Succinate	Lactate
1	3294	—	1277	631
2	—	1765	856	509

In another experiment (Table 5) the specific activities of the succinate and lactate produced when [^{14}C]propionate was metabolized in the presence of malonate were compared. Since lactate is oxidized by acid permanganate it was not possible to use this reagent to destroy malonate. The amounts of succinate and lactate relative to malonate were

therefore increased by adding unlabelled succinate and lactate, equivalent to ten times the quantities of these acids in the medium, before ether extraction, in order to avoid swamping of the peaks on chromatography.

The possibility that labelled succinate may be formed from propionate by some route other than direct carboxylation was checked by control experiments with other substrates which would be likely intermediates. Table 6 gives the results of an experiment with fumarate in which both the fumarate and carbon dioxide were labelled in separate runs. The specific activity of the succinate was much lower than that obtained from propionate. In the next experiment (Table 7) pyruvate was also included, but the activity of the succinate was no greater than in the control without substrate. Table 8 shows negligible fixation of carbon dioxide with acrylate, lactate or pyruvate as substrate.

Table 6. *Quantity and radioactivity of succinate formed from fumarate or propionate in presence of malonate*

Rumen epithelium (3 g.; from Institute sheep) was incubated for 3 hr. at 39·5° in Ringer–phosphate–bicarbonate containing sodium malonate (0·02 M) and the additions shown. Gas phase: $O_2 + CO_2$ (95:5, v/v).

	Succinate produced (μmoles)	Incorporation of labelled carbon into succinate (μatoms)	Incorporation of labelled carbon into succinate (% in additional succinate*)
No addition, $^{14}CO_2$	4·47	0·64	14
Na fumarate (500 μmoles), $^{14}CO_2$	11·94	1·84	16
Na [*carboxy*-^{14}C]fumarate (500 μmoles), CO_2	14·33	0·55	5
Na propionate (400 μmoles), $^{14}CO_2$	9·90	4·23	66

* Specific activity of succinate or additional succinate formed/specific activity of substrate. The assumption is made that endogenous succinate production is unaltered when fumarate or propionate is metabolized.

Table 7. *Quantity and radioactivity of succinate formed from pyruvate, fumarate or propionate in presence of malonate*

Rumen epithelium (3 g.; from Institute sheep) was incubated for 3 hr. at 39·5° with Ringer–phosphate–bicarbonate containing sodium malonate (0·02 M) and the additions shown (as sodium salts). Gas phase: $O_2 + CO_2$ (95:5, v/v).

	Succinate produced (μmoles)	Incorporation of labelled carbon into succinate (μatoms)	Incorporation of labelled carbon into succinate (% in additional succinate*)
No addition, $^{14}CO_2$	4·30	0·03	<1
Pyruvate (200 μmoles), $^{14}CO_2$	6·52	0·03	0
[*carboxy*-^{14}C]Fumarate (200 μmoles), CO_2	6·46	0·34	16
Propionate (400 μmoles), $^{14}CO_2$	11·29	3·04	44
[*carboxyl*-^{14}C]Propionate (400 μmoles), CO_2	8·30	1·93	48

* Specific activity of succinate or additional succinate formed/specific activity of substrate. It is assumed that the presence of the substrates does not alter the endogenous succinate production.

Table 8. *Quantity and radioactivity of succinate formed from lactate, acrylate, pyruvate, fumarate or propionate in presence of malonate*

Rumen epithelium (3 g.; from Institute sheep) was incubated for 3 hr. at 39·5° with Ringer–phosphate–bicarbonate containing sodium malonate (0·02 M) and the additions shown (as sodium salts). Gas phase: $O_2 + CO_2$ (95:5, v/v).

	Succinate produced (μmoles)	μatoms of labelled carbon in succinate	Percentage of succinate incorporating labelled carbon*
No addition, $^{14}CO_2$	3·34	0·13	4
Acrylate (400 μmoles), $^{14}CO_2$	4·15	0·01	—
Lactate (400 μmoles), $^{14}CO_2$	5·64	0·20	3
Pyruvate (400 μmoles), $^{14}CO_2$	4·48	0·12	—
[*carboxy*-^{14}C]Fumarate (500 μmoles), CO_2	9·88	0·26	4
[*carboxy*-^{14}C]Propionate (400 μmoles), CO_2	4·46	0·65	58

* Specific activity of succinate or additional succinate formed/specific activity of substrate. It is assumed that the presence of the substrates does not alter the endogenous succinate production.

Table 9. *Metabolism of* [carboxy-^{14}C]*propionate in in the presence of a succinate pool*

Rumen epithelium (2 g.; from slaughterhouse sheep) was incubated for 3 hr. at 39·5° with Ringer–bicarbonate and the additions shown. Gas phase: O_2+CO_2 (95:5, v/v).

Additions	Propionate carboxyl-C in CO_2 (μatoms)	Percentage of added radioactivity appearing in succinate	Succinate utilized (μmoles)	Fumarate formed (μmoles)	Malate formed (μmoles)	Lactate formed (μmoles)
100 μmoles of [*carboxy*-^{14}C]-propionate + 100 μmoles of succinate	34	<0·01	76·9	0	14·5	18·6
5 μmoles of [*carboxy*-^{14}C]-propionate + 200 μmoles of succinate	3·5	0	89·3	9·5	17·2	12·8
100 μmoles of succinate	—	—	68·6	8·9	9·5	5·3

Table 10. *Metabolism of propionate and pyruvate by sheep-rumen epithelium*

Tissue (2 g. from slaughterhouse sheep) was incubated for 3 hr. at 39·5° in Ringer–phosphate (gassed with O_2) or Ringer–phosphate–bicarbonate (gassed with O_2+CO_2, 80:20, v/v) and 100 μmoles of sodium propionate and/or 100 μmoles of sodium pyruvate, as indicated. All results are corrected to 100 mg. (dry wt.) of tissue.

Expt. no.	CO_2	Addition	Pyruvate used (μmoles)	Propionate used (μmoles)	Lactate formed (μmoles)	Ketone bodies formed (μmoles)
1	–	—	—	—	2·3	3·5
		Pyruvate	24·5	—	10·8	9·5
		Propionate	—	1·5	2·0	0·9
		Pyruvate + propionate	21·6	0·4	14·6	2·7
2	–	—	—	—	1·3	2·6
		Pyruvate	29·2	—	8·2	8·5
		Pyruvate + propionate	22·1	—	17·6	1·0
3	–	—	—	—	1·5	3·8
		Pyruvate	31·2	—	12·3	10·7
		Propionate	—	1·6	2·0	1·3
		Pyruvate + propionate	29·3	6·4	20·3	2·9
4	+	—	—	—	2·5	2·2
		Pyruvate	42·5	—	18·3	5·6
		Propionate	—	28·0	15·6	0·4
		Pyruvate + propionate	37·1	28·0	31·6	0·4
5	+	—	—	—	0·7	2·3
		Pyruvate	30·8	—	14·0	6·1
		Propionate	—	27·6	14·1	1·6
		Pyruvate + propionate	24·2	22·8	34·8	1·4

An alternative procedure to the use of inhibitors for trapping labelled intermediates is to metabolize the labelled substrate in the presence of a large pool of the unlabelled, suspected intermediate. The pool should become radioactive provided that the added and metabolically produced molecules are, to some degree at least, interchangeable within the system. Table 9 represents the results of an experiment in which [*carboxy*-^{14}C]propionate was metabolized in the presence of unlabelled succinate. In the last of the three runs shown succinate was metabolized alone, and labelled propionate added at the end of the incubation period to check that the succinate when isolated by chromatography possessed no radioactivity due to contamination with propionate.

Contrary to expectations the succinate pool had negligible activity at the end of the experiment. Two other peaks, in positions corresponding to fumarate and malate, appeared on the chromatogram when succinate was present so that the latter evidently penetrated the cells. The identity of the fumarate and malate was confirmed by co-chromatography and, in the case of malate, by fluorimetric assay (Hummel, 1949) of the fractions from the column.

It has been shown in another paper (Pennington & Sutherland, 1956) that when pyruvic acid is metabolized by rumen epithelium there is an increase in ketone-body formation. The fact that propionate suppresses endogenous ketone-body

formation in the tissue (Pennington, 1952) seemed also to rule out an oxidation pathway to pyruvate as postulated for liver by Huennekens *et al.* (1951), Mahler & Huennekens (1953), and favours conversion into a dicarboxylic acid. The experimental results given in Table 10 indicate that propionate also suppresses ketone-body formation from pyruvate when both are metabolized together. This is so even when, in the absence of carbon dioxide, hardly any propionate is metabolized.

DISCUSSION

It has been well established that, in animal tissues, propionate may be converted ultimately into carbon dioxide, lactate or carbohydrate (Deuel, Butts, Hallman & Cutler, 1935; Ringer, 1912; Huennekens *et al.* 1951; Daus, Meinke & Calvin, 1952). The formation of lactate and carbon dioxide by rumen epithelium accords with this. However, the route by which propionate enters the known metabolic pathways has been extremely hypothetical.

The present results appear to rule out a direct oxidative pathway to pyruvate as postulated by Huennekens *et al.* (1951). The incorporation of the carboxyl carbon of propionate or the carbon of carbon dioxide to such a high and similar degree into succinate (Table 3) can only be convincingly explained by the addition of carbon dioxide to propionate or a close derivative to form succinate (or succinyl-coenzyme A). Furthermore, such a direct pathway would not explain the loss of most of the radioactivity of the carboxyl group when propionate is converted into lactate (Table 1), and the correspondingly large incorporation of carbon dioxide into the carboxyl group of lactate (Table 2). Of particular importance is the fact that the specific activity of the lactate was lower than that of the succinate (Table 5).

The fact that the specific activity of the succinate isolated was less than that of the propionate may be explained as a result of the carboxylation of propionate together with the incomplete blocking of succinate oxidation by malonate (evidenced by the large amount of radioactive carbon dioxide formed) and the operation of the citric acid cycle in the tissue, for which some evidence has been provided (Pennington & Sutherland, 1956). The succinate leaking past the block will be converted into oxaloacetic and pyruvic acids and condensation of these will ultimately regenerate succinate. The carboxyl carbon of the original succinate will be lost during this chain of reactions and the newly formed succinate will be unlabelled.

It is possible to give a quantitative explanation of the lowering of the specific activity of the succinate if one accepts certain other assumptions which accord well with what is known of the metabolism of rumen epithelium. In the first place it is assumed that the carboxylation of propionate is irreversible; evidence for this was provided by the lack of radioactivity in the residual propionate when propionate was metabolized in the presence of $^{14}CO_2$. Further, that there must be no side-reactions (other than lactate formation) involving the acids of the citric acid cycle, and no piling up of intermediates other than succinate (and α-oxoglutarate, which will be included in the succinate). We have evidence which indicates that there is no important amino acid formation in this tissue. Thirdly, it is assumed that the succinate which leaves the active site of metabolism does not return. We have suggested elsewhere (Pennington & Sutherland, 1956) from consideration of other data that there may be restrictions on the movement of added dicarboxylic acids within this tissue.

Consequently, if x is the fraction of the succinate retained by the malonate block, a fraction $\frac{1-x}{2}$ will reappear as unlabelled succinate. On the second cycling $\left(\frac{1-x}{2}\right)x$ is retained and $\left(\frac{1-x}{2}\right)^2$ fresh succinate is formed, and so on. (It is assumed that the rate of reappearance of succinate and the rate of diffusion from the active site of metabolism are rapid compared with the duration of the experiment.)

If P is the amount of propionate metabolized the total succinate production will be, therefore:

$$Px\left[1+\left(\frac{1-x}{2}\right)+\left(\frac{1-x}{2}\right)^2+\dots\right]=\frac{Px}{1-\left(\frac{1-x}{2}\right)}=P\frac{2x}{1+x}.$$

The active succinate production will be Px.

Hence the specific activity of the succinate isolated relative to that of the first-formed succinate (and therefore the propionate) will be

$$\frac{Px}{P\dfrac{2x}{1+x}}=\frac{1+x}{2}.$$

A correction is necessary for lactate formation, which may be allowed for as follows:

If the amount of lactate formed is L, this represents a loss of $L\frac{x}{1+x}$ of unlabelled succinate. The total succinate formation will then be

$$(2P-L)\,x/(1+x),$$

and its specific activity relative to that of the propionate

$$\left(\frac{1+x}{2}\right)\left(\frac{P}{P-\frac{1}{2}L}\right).$$

In the experiments x is equal to the fraction of the isotope in the propionate metabolized which appears in the succinate. In Table 3 (column 7) the expected relative specific activity of the succinate, calculated as above, is given. There is good agreement with the experimental values. In Expts. 1 and 2 lactate formation was extremely low and was not corrected for.

The relatively low activity in the succinate formed from fumarate makes it improbable that carbon dioxide addition occurs after oxidation of propionate to acrylate (cf. Lardy & Fischer, 1953) or that succinate is formed by any other pathway ending in the reduction of fumarate.

The absence of any appreciable incorporation of propionate carboxyl carbon into a pool of succinate (Table 9) was surprising in view of the high isotope content of the succinate trapped by malonate. It seems necessary to postulate intracellular barriers to the ready interchange of produced and added succinate (see above).

In this connexion it is of interest that when *Escherichia coli* metabolized labelled acetate in the presence of added succinate or α-oxoglutarate, the labelled succinate formed apparently mixed readily with the trapping agent, but this was not so in the case of α-oxoglutarate (Swim & Krampitz, 1954; Ajl & Wong, 1954).

The formation of lactate from propionate could readily occur via succinate. Lactate is known to be formed from dicarboxylic acids by this tissue (Tables 2 and 9; see also Pennington & Sutherland, 1956) and presumably involves the decarboxylation of malate or oxaloacetate. Lactate formed thus from [*carboxy*-^{14}C]propionate should have a specific activity at most one-half that of the propionate. In actual fact (Table 1) the relative specific activity of the lactate was considerably less than this. This is probably a result of rapid interconversion of oxaloacetate and phosphoenolpyruvate which, together with rapid interconversion of oxaloacetate with fumarate, would lead to partial equilibration of the carboxyl-carbons of the acids with the carbon of the carbon dioxide pool in the flask.

Recently, several workers have studied the pathway of propionate metabolism in other tissues. Lardy and his co-workers have obtained evidence (Lardy & Peanasky, 1953) that in liver, also, propionate is converted into succinate by carboxylation, contrary to the mechanism postulated by Huennekens *et al.* (1951). A similar conclusion has been reached by Wolfe (1955). However, Katz & Chaikoff (1955) have shown that, in addition to succinate, *iso*succinate (methyl malonate) is formed from propionate by liver, indicating that both α- and β-carbons of propionate may be carboxylated. The formation of methyl malonate from propionate was discovered independently in Ochoa's laboratory (Flavin, 1955; Flavin, Ortiz & Ochoa, 1955). With pig-heart preparations, methyl malonate but no succinate was formed. Other tissues (rat heart, liver and kidney, sheep kidney) formed both products and were able to convert methyl malonate into succinate.

In our own experiments, methyl malonate, if formed by rumen epithelium, would have been destroyed during the removal of malonate with acid permanganate and have escaped detection.

Whiteley (1953) has demonstrated the reversibility of the decarboxylation of succinate in bacterial extracts.

The conversion of propionate into succinate seemed at first to be a likely explanation of the effectiveness of propionate in suppressing the endogenous ketone-body formation by this tissue. Further metabolism of the succinate would lead to the formation of oxaloacetate which, by condensing with acetyl-coenzyme A, would divert the latter away from acetoacetic acid formation. The results of Table 10, however, suggest another explanation for the antiketogenic activity of propionate. It may be seen that, as well as suppressing ketone-body formation from pyruvate, the presence of propionate also lowers the uptake of the latter. In contrast, glucose, which also suppresses ketone-body formation from pyruvate, actually increases the total quantity of pyruvate metabolized (Pennington & Sutherland, 1956). The experiments in this paper, which were all carried out with intact cells, throw no light on the intimate mechanism of the reaction, such as the possible involvement of coenzyme A. By analogy with other tissues, however, it is likely that the first step in propionate metabolism is the formation of propionyl-coenzyme A. By competing for available coenzyme A propionate may lower the concentration of acetyl-coenzyme A formed from pyruvate to a level where it may be all metabolized by condensation with oxaloacetate.

SUMMARY

1. When [*carboxy*-^{14}C]propionic acid was metabolized by sheep-rumen epithelial tissue, the greater part of the isotope appeared in carbon dioxide. Most or all of the remainder was found in the carboxyl group of the lactate formed; the specific activity of the lactate was much less than that of the propionate.

2. When propionate was metabolized, carbon dioxide was fixed into lactate. Relatively little carbon dioxide was fixed when pyruvate or fumarate was metabolized.

3. In the presence of malonate, succinate of high and similar relative specific activity was formed from propionate and carbon dioxide when either was labelled. Under similar conditions succinate

formed from pyruvate, fumarate, lactate or acrylate had relatively low specific activity. This is taken as evidence that the metabolism of propionate involves carbon dioxide fixation to form succinate.

4. There was negligible labelling of a succinate pool when labelled propionate was metabolized.

5. Fumarate and malate were produced from succinate by rumen epithelium.

6. Propionate suppressed ketone-body formation from pyruvate and lowered the total amount of pyruvate metabolized.

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A Comparison of the Action of Penicillinase on Benzylpenicillin and Cephalosporin N and the Competitive Inhibition of Penicillinase by Cephalosporin C

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A species of *Cephalosporium* (I.M.I. 49137) has been found to produce two hydrophilic antibiotics, named cephalosporin N and cephalosporin C (Abraham, Newton & Hale, 1954; Newton & Abraham, 1955). Cephalosporin N, a monoaminodicarboxylic acid which is highly sensitive to penicillinase and yields penicillamine (β-thiolvaline) and D-α-aminoadipic acid on hydrolysis, is probably (D-4-amino-4-carboxy-*n*-butyl)penicillin (Newton & Abraham, 1954). It appears to be identical with synnematin B (Abraham *et al.* 1955). Cephalosporin C is a monoaminodicarboxylic acid which is similar to cephalosporin N in chemical composition and also yields D-α-aminoadipic acid on hydrolysis. It resembles the penicillins in some of its properties but differs from them strikingly in others. For example, it is not sensitive to penicillinase and does not give β-thiolvaline on hydrolysis, but it does

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